PROCESS FOR PRODUCING GLUCOSE DEHYDROGENASES

TECHNICAL FIELD

The present invention relates to a process for producing glucose dehydrogenase. The glucose dehydrogenase that is obtained by this process can be suitably used in a glucose sensor.

BACKGROUND ART

Development of biosensors using enzymes that react specifically on specific substrates are actively under way regardless of the industrial field. A typical example of biosensors is the glucose sensor, which is mainly used in the medical field.

The glucose sensor serves to build a reaction system that contains an enzyme and an electron transmitter, and when using this glucose sensor, the glucose is quantitativley determined using, for example, the amperometric technique. Glucose oxidase (GOD) or glucose dehydrogenase (GDH) is used as the enzyme (Japanese Patent Application Laid-open No. 2002-65778).

Since GOD has high substrate specificity towards glucose and has excellent thermostability, massive production of the enzyme is possible, such that it has the advantage that the manufacturing cost is inexpensive compared to other

enzymes. However, systems that use GOD have the problem that they are easily influenced by the oxygen that is dissolved in the measurement sample, such that the dissolved oxygen exerts an effect on the measurement results.

On the other hand, systems that use GDH are not easily influenced by the oxygen that is dissolved in the measurement sample. Therefore, systems that use GDH can measure glucose concentration with good accuracy even when carrying out measurements in an environment where oxygen partial pressure is low, or when measuring high concentration samples that require large quantities of oxygen. However, GDH has the problems that its thermostability is bad and its substrate specificity is worse than GOD.

From such circumstances, an enzyme that complements the shortcomings of both GOD and GDH was sought. SODE, who is one of the present inventors, isolated a new strain (*Burkholderia cepatia* KS1 strain) from a soil in the neighborhood of a thermal spring and obtained a novel GDH from this strain as disclosed in International Patent No. WO02/36779. This GDH consisted of α, β and γ subunits (hereinafter referred to as "CyGDH"), its reaction rate with the electron transmitter was high, and had no problem in regards to heat resistance, such that it was suitable as an enzyme for use in a glucose sensor.

However, since productivity of CyGDH was bad in the KS1 strain, massive production of CyGDH by the KS1 strain was difficult when considering an industrial application.

When the present inventors therefore introduced the DNA coding for the α, β and γ subunits into *Escherichia coli* and expressed them, GDH was efficiently produced. However, this GDH consisted of the α, and γ subunits, which was missing the β subunit (hereinafter referred to as "αGDH"). As described, the total of α, β and γ subunits could not be expressed by the process of transforming *Escherichia coli*.

In addition, when the present inventors examined the characteristics of αGDH, αGDH was found to have a slower reaction rate with the electron transmitter compared to CyGDH, but higher heat resistance than CyGDH, and a smaller Km for glucose. That is to say, αGDH was identified to be as useful as CyGDH, as an enzyme for use in a glucose sensor.

In prior art, to prepare 2 species of enzyme that are useful in this way, it was necessary to carry out separately each of the acquisition of expression strain, culture and purification, which was disadvantageous from the perspective of manufacturing costs and efficiency.

DISCLOSURE OF THE INVENTION

An object of the present invention is to prepare efficiently 2 species of GDH, which may be applied in, for example, a glucose sensor and the like.

The process for preparing the glucose dehydrogenase pertaining to the present invention comprises introducing DNA containing the sequence described in SEQ ID NO.: 1 coding for the α subunit, which has a glucose dehydrogenase activity,

and the β subunit, which is an electron-transfer protein, into a microorganism belonging to the genus *Pseudomonas* to obtain a transformant, and culturing this transformant to produce a first glucose dehydrogenase containing the β subunit and a second glucose dehydrogenase not containing the β subunit.

The α subunit has a molecular weight of approximately 60kDa for example as determined by SDS-polyacrylamide gel electrophoresis under reducing conditions, is. On the other hand, the β subunit has a molecular weight of approximately 43kDa for example as determined by SDS-polyacrylamide gel electrophoresis under reducing conditions.

The DNA may contain a base sequence coding for the γ subunit which has a molecular weight of approximately 14kDa as determined by SDS-polyacrylamide gel electrophoresis under reducing conditions. In this case, the first and the second glucose dehydrogenase are produced as containing the γ subunit.

Examples of the microorganisms belonging to *Pseudomonas* include *Pseudomonas putida*, *Pseudomonas fluorescens*, *Pseudomonas aeruginosa* and the like; however, the use of *Pseudomonas putida* is preferred from the perspective of safety of the recombinant.

The DNA can be obtained from, for example, a microorganism that belongs to the genus *Burkholderia* that is capable of producing an enzyme having glucose dehydrogenase activity. The microorganism belonging to the genus

Burkholderia adopted in the present invention is not limited in particular as long as it is a microorganism that belongs the genus *Burkholderia* and is capable of producing the present enzyme; however, *Burkholderia cepatia*, in particular the *Burkholderia cepatia* KS1 strain (hereinafter, simply referred to as the "KS1 strain") is preferred.

This KS1 strain is a new strain that SODE isolated from a soil in the nationhood of a thermal spring, and was identified as *Burkholderia cepatia* from its microbiological properties, and named KS1 strain. This KS1 strain was deposited at The National Institute of Advanced Industrial Science and Technology International Patent Organism Depositary (Chuo Dai-6, 1-1, Higashi 1-chome, Tsukuba-shi, Ibaraki-ken, 305-8566 Japan) as Microorganism Depository No. FERM BP-7306, on September 25, 2000.

Note that when the present inventors measured glucose dehydrogenase activity in strains other than the KS1 strain by ordering a number of strains of the same *Burkholderia cepatia* that were deposited at the Institute for Fermentation, Osaka (IFO) or the Japan Collection of Microorganisms (JCM) of the Riken Institute of Physical and Chemical Research, they confirmed that the activity existed in all the strains. Therefore, *Burkholderia cepatia* other than the KS1 strain, for example, JCM5506, JCM5507, JCM2800, JCM2801, IFO15124 and IFO14595 can be adopted as the microorganism for obtaining the DNA used in the present invention.

The DNA is isolated from the chromosomal DNA of *Burkholderia cepatia*; however, since its base sequence and the amino acid sequence coded by the same base sequence are determined, it can also be obtained by chemically synthesis based on these sequences.

The amino acid sequence of SEQ ID NO.: 3, or an amino acid sequence wherein one or a plurality of amino acid residues have been substituted, deleted, intercalated or added in the amino acid sequence of SEQ ID NO.: 3, for example, is adopted as the α subunit. This α subunit is coded by, for example, the base sequence consisting of the bases No. 764 to 2380 among the base sequence of SEQ ID NO.: 1. Therefore, the use of a DNA having the above-mentioned base sequence is preferred as the DNA to be used in the present invention.

The amino acid sequence of SEQ ID NO.: 5, or an amino acid sequence wherein one or a plurality of amino acid residues have been substituted, deleted, intercalated or added in the amino acid sequence of SEQ ID NO.: 5, for example, is adopted as the β subunit. This β subunit is coded by, for example, the base sequence consisting of the bases No. 2386 to 3660 among the base sequence of SEQ ID NO.: 1. Therefore, the use of a DNA having the above-mentioned base sequence is preferred as the DNA to be used in the present invention.

It has been determined by SODE, that a high enzymatic activity is obtained when the γ subunit is expressed together with the α subunit compared to when the α subunit is expressed alone. Therefore, from the point of view of the enzymatic

activity, it is preferred to express the γ subunit, and it is preferred that in the DNA, the base sequence coding for the γ subunit be contained in the upstream region of the base sequence coding for the α subunit. It can be thought that, in this way, when producing the α subunit, the α subunit can be efficiently produced inside the body of the microorganism by the fact that the γ subunit is first expressed and is present as a protein.

The amino acid sequence of SEQ ID NO.: 2, or an amino acid sequence wherein one or a plurality of amino acid residues have been substituted, deleted, intercalated or added in the amino acid sequence of SEQ ID NO.: 2, for example, is adopted as the γ subunit. This γ subunit is coded by, for example, the base sequence consisting of the bases No. 258 to 761 among the base sequence of SEQ ID NO.: 1. Therefore, the use of a DNA having the above-mentioned base sequence is preferred as the DNA to be used in the present invention.

Among the base sequence of SEQ ID NO.: 1, it is supposed that the base sequence beyond base No. 2386 is coding for the β subunit, and it is speculated that the base sequence from base No. 2386 to 2451 is coding for the signal peptide of the β subunit. The amino acid sequence derived from the same signal peptide is the amino acid sequence from amino acid No. 1 to 22 of SEQ ID NO.: 4.

As the signal peptide is a peptide that is required when a protein that has been synthesized by the ribosome passes through the inner membrane and is secreted in the periplasmic

space, if the signal peptide is present, the quantity of protein contained in the periplasm of the cell or the culture supernatant is increased. Therefore, using a DNA containing the base sequence coding for the expression of the signal peptide of the β subunit as the DNA is preferred.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows the results of Q-Sepharose FF chromatography of a soluble fraction obtained from a transformant.

Fig. 2 shows the results of SDS-PAGE.

PREFERRED MODE FOR CARRYING OUT THE INVENTION

Hereinafter, the process for preparing the glucose dehydrogenase pertaining to the present invention will be described concretely.

A Step 1 of obtaining the DNA coding for the expression of for example the α subunit and the β subunit, a Step 2 of introducing a recombinant vector containing this DNA into a microorganism belonging to the genus *Pseudomona* to create a transformant, a Step 3 of culturing this transformant in a culture medium to produce a glucose dehydrogenase that contains the β subunit (for example CyGDH) and a glucose dehydrogenase that does not contain the β subunit (for example αGDH), and a Step 4 of collecting the glucose dehydrogenase from the culture medium or the microorganism, are included in the present preparation process.

<Step 1 (obtaining DNA)>

For obtaining the DNA, first, a recombinant vector is constructed. The recombinant vector is constructed, after isolating and purifying the chromosomal DNA from a microorganism belonging to the genus *Burkholderia* (for example *Burkholderia cepatia* KS1 strain), by linking and closing the chain between a chromosomal DNA fragment resulting from the cutting of this chromosomal DNA or a DNA fragment that was amplified by PCR and the like, and a linear expression vector.

Isolation and purification of the chromosomal DNA is carried out based on a bacterial lysate obtained from the bacteriolysis of microorganism. Example of bacteriolysis processs include, for example, treatment by a bacteriolysis enzyme such as lysozyme, and in addition to this treatment, proteases, other enzymes and detergents such as sodium lauryl sulfate (SDS) are used in combination, as necessary. In addition, physically rupturing processs such as freeze thawing and treatment with French press application can be used in combination. On the other hand, isolation and purification of DNA from the bacterial lysate can be carried out by suitably combining processs such as, for example, protein-removal treatments such as phenol treatment and protease treatment, ribonuclease treatment, and alcohol precipitation treatment.

Cutting of the chromosomal DNA can be carried out according to processs of the art, for example using sonication

treatment and restriction endonuclease treatment. As for the restriction endonuclease, for example, the type II restriction endonuclease that acts on a specific nucleotidic sequence is used.

Linkage of the chromosomal DNA fragment and the expression vector is carried out, for example, using DNA ligase.

Expression vectors that may self multiply inside the host microorganism and have been constructed for gene recombination from a bacteriophage or a plasmid are suitable. Examples of bacteriophages include, for example, Lambda gt10, Lambda gt11 and the like, in case *Escherichia coli* described below is to be the host microorganism. On the other hand, examples of plastids include, for example, pBR322, pUC18, pUC118, pUC19, pUC119, pTrc99A, pBluescript or SuperCosI, which is a cosmid, and the like, in case *Escherichia coli* is to be the host microorganism. In addition, examples include RSF1010, pBBR122, pCN51 and the like, which are wide host range vectors for Gram-negative bacteria, in case *Pseudomonas* is to be used.

Next, a marker is provided to the recombinant vector, and a host microorganism is transfected with this recombinant vector to create a transformant. Screening is carried out with the expression of this vector marker and the enzymatic activity as the index, to obtain the gene donor microorganism, which retains the recombinant vector containing the gene coding for GDH, from this transformant.

The host microorganism is not limited as long as the recombinant vector is stable, and self-replicating and can express the character of an exogenous gene. Generally, *Escherichia coli* DH5α, XL-1 Blue MR and the like can be used. For example in case the host microorganism is *Escherichia coli*, the competent cell process by calcium treatment, electroporation process, and the like can be used as processs for transfecting a host microorganism with a recombinant vector.

In addition, after the gene donor microorganism is cultured and the recombinant vector is isolated and purified from this microorganism, the gene coding for GDH (cloning fragment) is collected from the recombinant vector. Collection of the cloning fragment can be carried out by the same process as for collecting the chromosomal DNA.

This cloning fragment has a base sequence coding for the α subunit, which has the glucose dehydrogenase activity, and the β subunit, which is an electron-transfer protein. When obtaining the target DNA from the *Burkholderia cepatia* KS1 strain, the cloning fragment is obtained as one containing the base sequence coding for the γ subunit in addition to the base sequence coding for the α subunit and the β subunit (containing the signal peptide of the β subunit). Note that, the fact that the cloning fragment is coding for the α subunit and the β subunit, or the fact that it is coding for the γ subunit can be verified by decoding the base sequence of this cloning fragment by a process of the art.

<Step 2 (creation of the transformant)>

The transformant containing the target DNA is created by integrating the cloning fragment obtained in Step 1 into a vector, thereafter introducing the latter into a microorganism that belongs to the genus *Pseudomonas*. For example, *Pseudomonas putida* is used preferably as the microorganism that belongs to the genus *Pseudomonas*. The process for introducing a recombinant vector into a host microorganism can be carried out by the same process as for the creation of a transformant for the screening in Step 1.

<Step 3 (culture of the transformant and production of GDH)>

The transformant obtained in Step 2 is cultured so as to make it produce GDH. GDH containing the β subunit and GDH not containing the β subunit are simultaneously produced from this transformant. For example, in case the target DNA has been obtained from the *Burkholderia cepatia* KS1 strain, CyGDH having the α, β and γ subunits and αGDH having the α and γ subunits are produced simultaneously.

As far as the format of the culture of the transformant is concerned, it suffices to select the culture conditions by considering the nutritional physiological character of the host, and liquid cultures are often carried out. Industrially, it is advantageous to carry out aerobic shaking cultures.

As far as the source of nutrient for the culture medium is concerned, those that are generally used in cultures of

microorganism may be broadly used. As far as the carbon source is concerned, carbon compounds that can be assimilated are sufficient, and for example glucose, sucrose, lactose, maltose, molasses, pyruvic acid and the like are used. As for the nitrogen source, nitrogen compounds that can be assimilated are sufficient, for example peptone, meat extract, yeast extract, casein hydrolysate, soybean alkaline extract and the like are used. In addition, salts such as phosphate, carbonate, sulphate, magnesium, calcium, potassium, iron, manganese and zinc, specific amino acids, specific vitamins and the like are used as necessary.

The culture temperature may be suitably modified within a range where the transformant grows and the transformant produces GDH, and is preferably on the order of 20 to 42°C. It suffices that the culture completes at a suitable time by aiming for the time when GDH reaches maximum yield, and in general, culture time is on the order of 12 to 72 hours. The pH of the culture medium may be suitably modified within a range where the transformant developments and the transformant produces GDH, and preferably is in a range of on the order of pH 5.0 to 9.0.

<Step 4 (collection of GDH)>

Collection of GDH is carried out, in general, after isolating a GDH-containing solution from the culture solution or the transformant following a process of the art, by purifying this GDH-containing solution.

In the case where GDH is present inside the cell, the GDH-containing solution can be obtained, after collecting the cells from the culture solution by means such as filtration or centrifugal separation and the like, by disrupting these cells by a mechanical process or an enzymatic process such as lysozyme and adding a chelating agent such as EDTA and a detergent as necessary, to solubilize GDH. On the other hand, in the case where GDH is present outside of the cell (in the culture solution), it can be obtained by separating the culture solution and the cell by means such as filtration or centrifugal separation.

Purification of the GDH-containing solution can be carried out directly from this solution, or can also be carried out after concentrating the GDH in this solution. The concentration can be carried out, for example by vacuum concentration, membrane concentration, salt precipitation treatment, or fractional precipitation process by a hydrophilic organic solvent (for example methanol, ethanol and acetone). Heat treatment and isoelectric point treatment are also effective purification means for the concentration of GDH. Purification of the concentrated solution can be carried out, for example, by suitably combining gel filtration, adsorption chromatography, ion exchange chromatography and affinity chromatography. Thereby, GDH containing the β subunit and GDH not containing the β subunit can be obtained separately.

The purified enzyme obtained in this way can be powderized, for example, by lyophilization, vacuum drying and spray drying, and circulated in the market.

Example

In the following, concrete examples of the preparation process described above will be described while the fact that 2 species of GDH are obtained will be demonstrated through this example.

<Preparation of chromosomal DNA from the *Burkholderia cepatia* KS1 strain>

The chromosomal DNA from the *Burkholderia cepatia* KS1 strain was prepared following a process of the art. That is to say, first, the KS1 strain was agitated overnight at 34°C, using the TL liquid medium (polypeptone=10g, yeast extract=1g, NaCl=5g, KH_2PO_4=2g, glucose=5g; 1L total volume, pH 7.2). The grown cells were recovered with a centrifuge. These cells were suspended in a solution containing 10mM NaCl, 20mM Tris-HCl (pH8.0), 1mM EDTA, 0.5%SDS, and 100μg/mL proteinase K, and treated for 6 hours at 50°C. After the same quantity of phenol-chloroform was added to this suspension and agitated for 10 minutes at room temperature, the supernatant was recovered with a centrifuge. Sodium acetate was added thereto so as to obtain a final concentration of 0.3M, twice the amount of ethanol was overlaid to precipitate the chromosomal DNA at the middle layer. After the precipitate was fished out using a glass rod and washed with 70% ethanol, it was dissolved

in an appropriate amount of TE buffer, to obtain a chromosomal DNA solution.

<Creation of the transformant>

The full length of the Gene coding for GDH was amplified by the PCR process with the previously obtained chromosomal DNA as the template. The sequence of the N-terminal portion of the GDH γ subunit and the sequence of the C-terminal portion of the GDH β subunit were used as primers. After a fragment resulting from the cut of this DNA fragment at the restriction endonuclease recognition site located at the ends of each primer, and a DNA fragment containing the trc promoter were prepared and linked to the wide host range vector RSF1010, the vector was introduced into *Escherichia coli* JM109, to form a colony on an LB agar medium containing 50μg/ml streptomycin. A plasmid was prepared from the colony that developed, with this as the template and using the previously described PCR primer, a clone for which a fragment of approximately 3.4Kb is amplified by PCR was selected, which was introduced in the *Pseudomonas putida* ATCC47054 strain, to obtain the target transformant (GDH expressing strain).

<Culture of the transformant and generation production of GDH>

The culture of the transformant was carried out under aerobic culture conditions. More specifically, the culture of the transformant was carried out for 8 hours at 34°C, using 7L of a culture medium that was adjusted so as to have a composition per liter of culture solution of Table 1. Cells

were obtained by centrifuging 7L of the present culture solution at 9,000×g (4°C, 10 minutes).

TABLE1: COMPOSITION OF CULTURE MEDIUM

Polypeptone	10g
Yeast extract	1g
NaCl	5g
KH_2PO_4	2g
Glucose	5g
Einol (ABLE Co. Tokyo Japan)	0.14g
Total, distilled water	1L
pH adjustment	7.2

<Purification of GDH>

The cells that were obtained were disrupted, while in a dispersed state in 10mM potassium phosphate buffer solution (pH6.0), by applying a pressure difference of 1,500Kg/cm^2 with a French press (Otake Seisakusyo Co., Ltd., Tokyo, Japan). The cell extract that was obtained at this time was centrifuged for 10 minutes at 8,000×g, and a crude enzyme solution devoid of cell solids was obtained.

Triton-X100 was added to the crude enzyme solution so as to obtain a final concentration of 1%. Next, the solution was agitated slowly overnight at 4°C. Then, after ultracentrifugation (4°C, 69800×g, 90 minutes), it was re-centrifuged (4°C, 15000×g, 15 minute), and the solubilized fraction was obtained as the supernatant.

After this solubilized fraction was dialyzed against a 10mM potassium phosphate buffer solution (pH8.0) containing 0.2% Triton-X100, this solution was supplied to a Q-Sepharose

FF column (22mm ID×20cm Amersham Bioscience), which was equilibrated with a 10mM potassium phosphate buffer solution (pH8.0) containing 0.2% Triton-X100. The protein was eluted with a linear gradient such that the concentration of NaCl in the 10mM potassium phosphate buffer solution (pH8.0) goes from 0 to 500mM. The elution was carried out at a flow rate of 5mL/min.

In regard to the eluate, GDH activity was measured for each fraction. The result is represented schematically in Fig. 1. As can be understood from Fig. 1, two large peaks were identified within the range where the gradient was applied.

Measurement of GDH activity was carried out by following the reaction of reduction of the electron acceptor, which is based on the dehydrogenation of glucose. 2,6-dichlorophenolindophenol (DCIP) and phenazine methosulfate (PMS) were used as electron acceptors. The reaction was carried out in a polyethylene tube at a prescribed temperature.

First, 5μL of enzyme solution was added to 20μL of 25mM tris-hydroxymethyl-aminomethane HCl buffer solution (pH8.0) containing 0.75mM PMS and 0.75mM DCIP to prepare a mix solution. Beforehand, this mix solution was let to stand at a constant temperature for 1 minute. The reaction was started by adding 1μL of 2M glucose (final concentration: 77mM) to the mix solution, which was let to stand for 2 minutes at constant temperature. Next, 100μL of ice-cold distilled water or 120μL

of 7.5M urea was added to cool the sample. For this sample, bleaching based on DCIP reduction was measured chronologically using an ultramicro measurement cell (100μL) and a spectrophotometer that can perform measurements using this cell (UV160, Shimadzu, Kyoto. Japan). The measurement wavelength was set to 600nm, the absorption wavelength of DCIP. The molar extinction coefficient of DCIP was 22.23mM×cm^{-1}. One enzyme unit (U) was defined as the quantity that oxidizes 1μM glucose per minute under standard assay conditions. The protein concentration was measured with Lowry's process.

Next, fractions were collected separately for the two GDH activity peaks, dialyzed overnight with a 10mM potassium phosphate buffer solution (pH8.0, 4°C) containing 0.2% Triton-X100, and 2 species of GDH solution were prepared.

Each GDH preparation solution was purified separately using a DEAE-5PW column (8.0mm ID×7.5cm Toso, Tokyo, Japan). The column was pre-equilibrated with a 10mM potassium phosphate buffer solution (pH8.0) containing 0.2% Triton-X100. The protein was eluted at a flow rate of 1mL/min with a linear gradient such that the concentration of NaCl in the 10mM potassium phosphate buffer solution (pH8.0) went from 0 to 400mM. For each chromatography, the fractions with the highest GDH activity were collected, desalted overnight with a 10mM potassium phosphate buffer solution (pH8.0) containing 0.2% Triton-X100, and 2 species of purified enzyme (hereinafter referred to as "first purified enzyme" and "second purified enzyme" for convenience) were obtained.

<Determination of the subunits of the purified enzymes>

Each purified enzyme solution was subjected to electrophoresis by SDS-PAGE, and the molecular weights of the subunits were determined. The SDS-PAGE was carried out in an 8-25% polyacrylamide gradient gel using a Tris-Tricine buffer solution. The proteins on the gel were subjected to coomassie staining. Separation and development were carried out automatically with the Phast System (Pharmacia). The molecular mass was measured by the relative mobility of standard proteins.

The results from the SDS-PAGE electrophoresis are shown in Fig. 2. In Fig. 2 are respectively shown the coomassie staining of standard molecular weight marker proteins in lane 1, the coomassie staining of the first purified enzyme in lane 2, and the coomassie staining of the second purified enzyme in lane 3. As can be understood from the same Fig., the first purified enzyme was divided into proteins whose the molecular weight are approximately 60kDa, approximately 43kDa and approximately 14kDa. Therefore, it is suggested that the α subunit with a molecular weight of approximately 60kDa, the β subunit with a molecular weight of approximately 43kDa, and the γ subunit with a molecular weight of approximately 14kDa are bound in the first purified enzyme. The second purified enzyme was divided into proteins whose molecular weight are approximately 60kDa and approximately 14kDa. Therefore, it is suggested that the α subunit with a molecular weight of approximately 60kDa and the γ subunit

with a molecular weight of approximately 14kDa are bound in the second purified enzyme. The results of the SDS-PAGE electrophoresis show that the first purified enzyme and the second purified enzyme are different GDH, and that 2 species of GDH were simultaneously generated.

After the bands containing the α subunit and the β subunit by electrophoresis were respectively cut out and transferred onto poly vinylidene fluorid membranes, the amino acid sequence was analyzed with an amino acid sequencer (Shimadzu, PPSQ-10).

For the α subunit, it was determined that it contained a peptide sequence of 11 residue consisting of the amino acid No. 2 to 12 in the amino acid sequence of SEQ ID NO.: 3. On the other hand, for the β subunit, it was possible to identify an amino acid sequence of 16 residues at the N-terminus, shown in SEQ ID NO.: 5.

<Analysis of the recombinant vector>

The target recombinant vector was extracted from the transformants of *Pseudomonas putida* having GDH activity. The base sequence of the inserted DNA fragment of this recombinant vector was determined by a process of the art. The result showed that it contained the base sequence of SEQ ID NO.: 258 to 3660.

As disclosed in International Patent Publication No. WO02/36779 and the like, the base sequence coding for the α subunit is the base sequence of base No. 764 to 2380 among the base sequence of SEQ ID NO.: 1, the base sequence coding

for the β subunit is the base sequence of base No. 2386 to 3660 among SEQ ID NO.: 1, the base sequence coding for the γ subunit is the base sequence of base No. 258 to 761 among the base sequence of SEQ ID NO.: 1, and the base sequence coding for the signal peptide of the β subunit is the base sequence of base No. 2386 to 2451 among the base sequence of SEQ ID NO.: 1.

Therefore, it was verified that the target recombinant vector contains a base sequence coding for the α subunit, the β subunit (containing the signal peptide of the β subunit) and the γ subunit. Note that the amino acid sequence corresponding to each base sequence is shown in SEQ ID NO.: 3 for the α subunit, SEQ ID NO.: 5 for the β subunit, SEQ ID NO.: 2 for the γ subunit, and amino acids No. 1 to 22 among the amino acid sequence of SEQ ID NO.: 4 for the signal peptide of the β subunit.

As can be understood from the foregoing, in the present invention, it is possible to produce simultaneously, moreover, efficiently, 2 species of GDH, such as, for example, αGDH and CγGDH.